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Quantifying *Phytophthora medicaginis* in Susceptible and Resistant Alfalfa with a Real-Time Fluorescent PCR Assay

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Abstract

A real-time fluorescent PCR assay using a set of specific primers and a fluorochrome-labelled probe (TaqMan) was developed to quantify the amount of *Phytophthora medicaginis* DNA in alfalfa plants that were classified as either resistant or susceptible to the pathogen based on visual assessment of disease response. The assay clearly discriminated among three standard check alfalfa populations with different levels of resistance based on the analysis of DNA extracted from the roots of bulked plant samples. In two independent experiments, the Spearman rank correlation between pathogen DNA content and the number of susceptible plants in a bulked sample was greater than 0.89 and highly significant ($P < 0.0001$). Significantly less pathogen DNA was detected in bulked samples of a highly resistant check population than in bulked samples from more susceptible check populations. Analysis of individual plants indicated that significantly less pathogen DNA was detected in resistant plants than in susceptible plants. Applications of the assay are considered for breeding programs and the study of microbial population dynamics in plants simultaneously infected with different pathogens.

Introduction

Phytophthora medicaginis Hansen et Maxwell (=*Phytophthora megasperma* Drechs. f. sp. *medicaginis* T. Kuan & D. C. Erwin) is an important causal agent of root rot in both alfalfa (*Medicago sativa* L.) and chickpeas (*Cicer arietinum* L.). Root rot of alfalfa caused by *P. medicaginis* occurs in nearly every region of the world where alfalfa is cultivated (Erwin, 1990; Hansen and Maxwell, 1991). The disease can cause complete failure of stand establishment due to damping-off and seedling death, and is most prevalent when newly seeded fields are subjected to flooding (Havey and Grau, 1985; Erwin, 1990). The pathogen also causes disease in mature plants (Erwin, 1966; Leuchen et al., 1976). *P. medicaginis* produces oospores, which can survive for years in the soil (Erwin, 1990). Phytophthora root rot of alfalfa is best controlled by a combination of practices including cultivation of resistant varieties, water management, and use of the fungicide metalaxyl as a seed treatment (Erwin, 1990).

Various studies have indicated that alfalfa cultivars with resistance to *P. medicaginis* have significantly higher yields than susceptible cultivars when grown in naturally infested soil (Faris and Sabo, 1981; Gray et al., 1988). The percentage of plants exhibiting either adult plant resistance (Barnes and Frosheiser, 1983) or seedling resistance (Faris, 1985) to *P. medicaginis* can vary considerably among alfalfa cultivars that are considered resistant. A significant positive correlation ($r = 0.78$) has been reported between levels of resistance obtained from a greenhouse seedling assay and resistance ratings obtained from field grown mature plants (Hohrein et al., 1983).

Most recently released alfalfa cultivars have at least a moderate degree of resistance to *P. medicaginis* (Alfalfa Council, 2002). However, the disease still causes considerable losses in alfalfa production because many growers continue to cultivate less expensive older cultivars that lack disease resistance (Munkvold and Carlton, 1995). In addition, *P. medicaginis* has been implicated in damping-off disease complexes of alfalfa with other soilborne pathogens including the northern root knot nematode (*Meloidogyne hapla* Chitwood) (Gray et al., 1990; Griffin and Gray, 1994), southern root knot nematode [*Meloidogyne incognita* (Kofoid & White) Chitwood] (Welty et al., 1980) and *Aphanomyces euteiches* (Havey and Grau, 1985; Munkvold and Carlton, 1995).

Several different mechanisms have been implicated in the expression of resistance in alfalfa to *P. medicaginis*, including hypersensitive reactions in the root cortical cells of resistant plants (Marks and Mitchell, 1971; Vaziri et al., 1981), and increased production in

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resistant plants of the phytoalexin medicarpin (Vaziri et al., 1981). Hyphal staining of infected roots of both alfalfa (Miller and Maxwell, 1984) and chickpea (Dale and Irwin, 1991) has indicated that colonization by *P. medicaginis* is more extensive in susceptible plants than in resistant plants. However, it is difficult to use hyphal staining techniques to accurately quantify difference between root systems in the amount of colonization by *P. medicaginis*. Entire root systems cannot be stained on a single slide, and hyphae do not typically grow in a linear manner, making measurement difficult.

Comparisons have not been made between resistant and susceptible alfalfa plants for the number of oospores of *P. medicaginis* produced in infected roots, but these comparisons have been done for other *Phytophthora* sp. Significantly more oospores of *P. fragariae* were produced in roots of susceptible strawberry plants than in resistant plants (Milholland et al., 1989). However, no significant differences were observed in the number of oospores of *P. infestans* produced on leaves of potato genotypes having different levels of resistance to potato late blight disease (Cohen et al., 1997). Counting oospores is a tedious process, and entire root systems cannot be sampled easily.

The development of techniques for real-time detection of fluorescent-labelled DNA fragments amplified using polymerase chain reaction (PCR) may provide another approach for accurately quantifying the amount of *P. medicaginis* in infected plants (Holland et al., 1991; Livak et al., 1995; Heid et al., 1996; Lie and Petropoulus, 1998). Real-time detection of PCR products (amplicons) employs a chemistry that uses the 5′ nuclease activity of *Taq* Polymerase to generate fluorescence in proportion to the number of target amplicons produced (Holland et al., 1991). After an initial period, successive cycles of PCR result in the exponential synthesis of the amplicon, which can be quantified automatically using software that measures increase in fluorescence (Schoen et al., 1996).

Recently, a real-time PCR assay was developed for *A. euteiches* (Vandemark et al., 2002). Spearman rank correlations between pathogen DNA content and disease severity index (DSI) ratings were greater than 0.75 and highly significant ($P < 0.0005$) for analysis of both individual alfalfa plants and bulked plant samples of resistant and susceptible alfalfa check populations (Vandemark et al., 2002). The highly positive and significant correlations between pathogen DNA quantity and DSI suggested it may be possible to use real-time PCR to select the most resistant plants from among a sample of plants considered to be equally resistant based on a visual assessment of disease severity.

Our objectives were: (1) develop a real-time quantitative PCR assay that was specific for *P. medicaginis*, (2) employ the assay to discriminate between standard check alfalfa populations for resistance to *P. medicaginis* by analysis of DNA extracted from bulked plant samples, and (3) use the assay to compare differences between resistant and susceptible plants in the amount of colonization of entire root systems by *P. medicaginis*. Applications of this assay for breeding programs and the study of microbial population dynamics are considered.

Materials and Methods

Pathogen and plant materials

Phytophthora medicaginis isolate WI301 was used in this study for all infectivity assays. The isolate was originally obtained from infected alfalfa plants grown under field conditions in Wisconsin. The isolate was maintained on potato dextrose agar (PDA, Difco Inc., Sparks, MD, USA) at 20–24°C. Three different standard check alfalfa populations were evaluated: the varieties Saranac and Agate, and the germplasm WAPH-1. Saranac is the susceptible check in standard tests for evaluating resistance to *P. medicaginis*, Agate is a resistant check, and WAPH-1 is the highly resistant check (Nygaard et al., 1995).

Inoculations and evaluation of disease severity

Phytophthora medicaginis isolate WI301 was used to examine the ability of a real-time fluorescent PCR assay to discriminate check populations for resistance based on the analysis of DNA extracted from the roots of bulked plant samples. The standard test protocol for evaluating seedling resistance in alfalfa to *P. medicaginis* was closely followed in this study (Nygaard et al., 1995), except that each plant was inoculated with 250 zoospores per plant instead of 50 zoospores. This was done to minimize the likelihood of plants escaping infection.

Alfalfa seeds were mixed in a commercial preparation of *Sinorhizobium mellioti* (LiphaTech Inc., Milwaukee, WI), and planted in plastic greenhouse flats containing vermiculite. A single row of each of the three check alfalfa populations was sown in each flat. Seedlings were grown in the greenhouse with 16 h daylength at 20–24°C. The rows were thinned to approximately 70 seedlings/row 8 days after sowing. Cultures of isolate WI301 were transferred from PDA plates to plates containing V-8 agar and zoospores were produced as previously described (Miller and Maxwell, 1984). At 10 days post-sowing, the vermiculite was saturated with water and each seedling was inoculated with 1 ml of a 250 zoospore/ml suspension by pipetting the suspension to the base of the stem. The flats were flooded for 2 days. Ten days after inoculation, 15 plants were randomly selected from each row and rated for resistance as follows: resistant = vigorously growing plants with slight to no necrosis of tap and secondary roots; hypocotyls area sound with slight to no chlorosis of cotyledons, or susceptible = stunted or dead plants with moderate to severe necrosis of roots, hypocotyls and cotyledons (Nygaard et al., 1995). For each sample of 15 plants the number of resistant and susceptible plants was counted and the entire sample was bulked. DNA was subsequently isolated from each entire bulked plant sample and analyzed by quantitative PCR.

The experiment consisted of eight replicate flats and the experiment was repeated once. For each experiment a non-inocululated flat was also maintained that contained the three standard check alfalfa populations for a source of DNA from uninfected seedlings, which served as controls in quantitative PCR analysis. These flats were maintained on different greenhouse benches than the flats containing infected plants. Uninoculated plants of each population were bulked into groups of 15 plants prior to DNA extraction.

An additional experiment was conducted to examine the relationship between disease severity and the amount of pathogen DNA detected in roots of individual alfalfa plants. In this experiment, a flat was planted with a single row of the highly resistant check population WAPH-1. Inoculum preparation, plant inoculations and rating of plants as either resistant or susceptible were conducted as described above. Twelve resistant and twelve susceptible plants were randomly selected and DNA was subsequently isolated from each individual plant and analysed by quantitative PCR. The experiment was repeated once.

DNA extraction

The plants of each bulk sample were placed with their hypocotyls together, rinsed in tap water, blotted dry on sterile paper towels and 200 mg of root tissue closest to the mass of hypocotyls was excised with a razor blade. DNA was extracted from the bulk sample using the Fast-DNA kit (Q-Biogene, Inc., Carlsbad, CA, USA) according to manufacturer's recommendations. DNA was extracted from the entire root sample of individual plants as described above. DNA was also isolated as previously described (Vandemark et al., 2000) from a pure culture of *P. medicaginis* WI301 grown in potato dextrose broth (PDB; Difco Inc., Detroit, MI, USA). DNA was quantified with a fluorometer (TD-700; Turner Designs, Inc., Sunnyvale, CA, USA), and diluted to 20 ng/μl for use in quantitative PCR reactions.

PCR primer and probe design

We previously identified a sequence characterized DNA marker (SCAR) that was only amplified in isolates of *P. medicaginis*. The 1141 bp DNA sequence (GenBank Accession no. AY251053) corresponding to this SCAR marker was analyzed using Primer Express software (Applied Biosystems, Foster City, CA, USA) to identify nucleotide sequences of the forward primer, reverse primer and fluorochrome-labelled probe used in this study. The forward primer p990F (5′-d-GGTGGGTGGAACGAAGGA-3′), reverse primer p1050R (5′-d-TGGCAGCGGAGATCCAA-3′), and probe p1010CT (5′-CCGCGCCAGTATTTGTCTTCC GG-3′) were commercially synthesized (Applied Biosystems). The 5′ terminus of the probe (TaqMan; Applied Biosystems) was labelled with the fluorochrome 6-carboxyfluorescein (6FAM) and the 3′ terminus labelled with the quencher dye tetra-methylcarboxy-rhodamine (TAMRA). The primer/probe set p990F-p1010CT-p1050R amplified a 61 bp fragment. Primers were stored at −20°C as 90 μM stocks in ddH_2O and the probe was stored, protected from light, at −20°C.

Quantitation of *P. medicaginis* DNA in infected plants

DNA isolated from individual plants and from bulked plant samples, both infected and healthy, was analyzed using the primer/probe set p990F-p1010CT-p1050R. For each DNA sample, four replicate reactions were run in 50 μl reactions containing 100 ng of DNA, 900 nM forward primer p990F, 900 nM reverse primer p1050R, 150 nM probe p1010CT, 5 μl ddH_2O, and 25 μl of 2X TaqMan Universal PCR Master Mix (Applied Biosystems). Amplifications and detection of fluorescence were done using a GeneAmp 7000 Sequence Detection System (Applied Biosystems). The thermocycling profile for all PCR reactions was the manufacturer's suggested default cycling profile, which consists of an initial cycle of 2 min at 50°C, then a single cycle of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The concentrations of primers and probes listed above were determined through a series of optimization reactions for which a matrix of different primers and probe concentrations were tested in replicated PCR reactions using purified genomic DNA of *P. medicaginis* WI301. Optimal forward and reverse primer concentrations were chosen that resulted in the maximum normalized reporter fluorescence (ΔR_N). The optimal TaqMan^{TM} probe concentration was chosen that resulted in the minimal cycle threshold (C_T).

For the quantification of *P. medicaginis* WI301, standard curves were constructed by including reactions containing pure DNA of isolate WI301 that spanned eight different initial DNA quantities per reaction (0.001, 0.01, 0.1, 1.0, 5, 25, 50, and 100 ng). Each analysis included three replicate reactions for each DNA quantity. Reactions containing 100 ng DNA isolated from uninfected Saranac, Agate, and WAPH-1 were also included to confirm that the primer/probe sets selectively amplified DNA from the pathogen genome and not from plant DNA. Each analysis also included a non-template control reaction, in which ddH_2O was substituted for DNA to confirm that the reagents were free of contaminating template DNA. The specificity of the primer/probe set towards *P. medicaginis* was also verified by attempting to use the primer/probe set to amplify DNA from several Oomycete plant pathogens and fungal species known to cause root rot in alfalfa and other legumes (Table 1).

All of the isolates listed in Table 1, except *P. infestans*, were grown in potato dextrose broth for 5 days at 20–24°C. DNA was extracted as previously described (Vandemark et al., 2000) from each culture, quantified with a fluorometer (TD-700; Turner Designs, Inc., Sunnyvale, CA, USA), and diluted to 20 ng/μl for use in quantitative PCR reactions. One-hundred nanogram of purified DNA was used as template for real-time PCR assays in 50 μl reactions as described above. DNA of *P. infestans* was provided by

Table 1
Specificity of the primer/probe set 990F-1010T-1050R towards *Phytophthora medicaginis* DNA[1]

Isolate	Host/origin	Equation of standard curve[2]
P. medicaginis FD1206	Alfalfa/WI	$Y = -3.22X + 23.3$; $R^2 = 0.99$
P. medicaginis N25	Alfalfa/WI	$Y = -3.20X + 23.2$; $R^2 = 0.99$
P. medicaginis WI301	Alfalfa/WI	$Y = -3.07X + 23.1$; $R^2 = 0.99$
P. medicaginis IL8	Alfalfa/IL	$Y = -3.25X + 23.0$; $R^2 = 0.99$
P. medicaginis N2019	Alfalfa/WI	$Y = -3.04X + 25.2$; $R^2 = 0.99$
Aphanomyces euteiches MF1	Alfalfa/WI	NA
A. euteiches NC1	Alfalfa/NC	NA
P. infestans	Potato/NY	NA
Pythium aphanidermatum	Bean/CA	NA
Pythium ultimum	Bean/WI	NA
Rhizoctonia solani	Pea/WA	NA
Thelaviopsis basicola	Pea/WA	NA
Mycosphaerella pinoides	Pea/OR	NA
Fusarium solani	Pea/WA	NA
F. oxysporum	Pea/OR	NA

[1]Initial amplifications were performed using 100 ng genomic DNA as template.
[2]Standard curves based on three replicated reactions per each known quantity (0.1, 1, 5, 25, 50, and 100 ng) of purified genomic DNA. R^2 values calculated for relationship between: Y, cycle threshold (C_T); X, $\log_{10}$ quantity of DNA (ng). NA, Not applicable.

H. Judelson (University of California, Riverside, CA, USA). Standard curves were constructed with DNA of all tested isolates of *P. medicaginis* by including reactions containing DNA that spanned eight different initial DNA quantities per reaction (0.001, 0.01, 0.1, 1.0, 5, 25, 50, and 100 ng).

Data analysis

For each experiment, data from the amplification of DNA from eight bulked samples for each population was subjected to an analysis of variance and a pairwise *t*-test using JMP Statistical Discovery Software (SAS Institute, Cary, NC, USA) to determine if differences in the amount of detected pathogen DNA were significant ($P \leq 0.05$). For each experiment, the Spearman rank correlation (Ostle, 1954) was calculated between the number of susceptible plants in a bulked sample and the mean of four replicate quantitative PCR reactions for each bulk sample. For experiments with individual plants, data from the amplification of DNA samples from resistant and susceptible in plants was subjected to an analysis of variance and a pairwise *t*-test to determine if differences in the amount of detected pathogen DNA were significant ($P \leq 0.05$) between phenotypic classes.

Results

Primers/probe set sensitivity and specificity

The primer/probe set p990F-p1010CT-p1050R could detect *P. medicaginis* DNA in very linear assays within a range of 0.001–100 ng initial quantity. The results were very similar for all tested isolates of *P. medicaginis*, with the correlation between the $\log_{10}$ of the initial DNA quantity and the C_T value ranging from −0.995 to −0.999. The primer/probe set did not amplify DNA from any other pathogens tested, including several other Oomycete plant pathogens (Table 1). In addition, the primer/probe set did not amplify DNA from non-inoculated plants of any of the three standard check alfalfa populations.

Discriminating between alfalfa standard check populations for reaction to *P. medicaginis*

In both experiments, analysis of variance indicated that significant differences were observed for the amount of DNA of *P. medicaginis* WI301 detected in bulked plant samples of the susceptible check Saranac, the resistant check Agate, and the highly resistant check WAPH-1 ($P < 0.0001$). In both experiments, significant differences ($P < 0.0005$) were observed for the amount of pathogen DNA detected in bulk plant samples within a given check population (population × bulk effects).

Results of comparisons of means between the three check populations for the amount of pathogen DNA detected in roots of bulked samples are presented (Table 2). In both experiments significantly less pathogen DNA was detected in the highly resistant check WAPH-1 than was detected in the two other check populations, and significantly less DNA was detected in the resistant check Agate than was detected in the susceptible check Saranac. The Spearman rank correlation between the number of susceptible plants in a bulk sample and the amount of *P. medicaginis* WI301 DNA detected in the bulk sample was positive and significant for both experiments (Table 2).

Relationship between disease reaction and quantity of pathogen DNA detected in individual plants

In both experiments, ANOVA indicated that significant differences existed between resistant and susceptible

Table 2
Comparison of means between bulked plant samples of alfalfa standard check populations for quantity (ng) of *Phytophthora medicaginis* DNA[1]

Population[2]	DNA (ng) experiment 1	DNA (ng) experiment 2
WAPH-1	0.31 a	0.21 a
Agate	1.31 b	1.43 b
Saranac	2.29 c	3.91 c
LSD ($\alpha = 0.05$)	0.13	0.31
ρ ($P > \lvert\rho\rvert$)	0.90 (< 0.0001)	0.94 (< 0.0001)

[1]Each experiment included eight bulks of 15 plants each for each population. Prior to bulking, plants were individually classified as either resistant (vigorously growing plants with slight to no necrosis of tap and secondary roots; hypocotyls area sound with slight to no chlorosis of cotyledons), or susceptible (stunted or dead plants with moderate to severe necrosis of roots, hypocotyls and cotyledons). DNA was extracted from roots of bulked plant samples and tested using the primer/probe set p990F-p1010CT-p1050R. Each bulk DNA sample was tested with four replicate PCR reactions. The Spearman rank correlations (ρ) between pathogen DNA quantity and the number of susceptible plants in a bulk sample are presented.
[2]WAPH-1, highly resistant; Agate, resistant; Saranac, susceptible; LSD, least significant difference.

Table 3
Comparisons of means for quantity (ng) of *Phytophthora medicaginis* DNA detected in resistant and susceptible plants[1]

	Experiment 1		Experiment 2	
Phenotype	DNA (ng)	Range	DNA (ng)	Range
Resistant	0.05 a	0–0.22	0.22 a	0–0.62
Susceptible	2.04 b	1.21–2.43	3.81 b	1.09–7.62
LSD ($\alpha = 0.05$)[2]	0.11		0.52	

[1]Each experiment included 12 resistant plants and 12 susceptible plants of the standard check alfalfa population WAPH-1. Resistant = vigorously growing plants with slight to no necrosis of tap and secondary roots; hypocotyls area sound with slight to no chlorosis of cotyledons, and susceptible = stunted or dead plants with moderate to severe necrosis of roots, hypocotyls and cotyledons. DNA was extracted from the entire root system of each plant and tested using the primer/probe set p990F-p1010CT-p1050R. Each plant was tested with four replicate PCR reactions.
[2]LSD, least significant difference.

plants for the amount of pathogen DNA detected ($P < 0.0001$). ANOVA was also performed separately for both phenotypic classes. Significant differences ($P < 0.0001$) were observed in the amount of pathogen DNA detected among resistant plants in both experiments. Similarly, in both experiments significant differences ($P < 0.0001$) were observed in the amount of pathogen DNA detected among susceptible plants.

A comparison of means for the amount of pathogen DNA detected in resistant and susceptible plants is presented (Table 3). In both experiments, significantly less pathogen DNA was detected in resistant plants than was detected in susceptible plants. The ranges for the amount of pathogen DNA detected in plants of both phenotypic classes are presented (Table 3). In both experiments, pathogen DNA was detected in all susceptible plants. In the first experiment, no pathogen DNA was detected in 16.7% (two of 12) of the resistant plants. In the second experiment, no pathogen DNA was detected in 41.7% (five of 12) of the resistant plants.

Discussion

Standard curves were generated for primer/probe set p990F-p1010CT-p1050R using DNA isolated from five different isolates of *P. medicaginis*. Correlations between initial DNA quantity and C_T value exceeded −0.99 for all isolates (Table 1). These results demonstrate the extremely precise quantification of target DNA that is possible using this real-time fluorescent PCR assay. The primer/probe set did not amplify DNA from healthy plants of the three alfalfa check populations nor from any other pathogen tested, including several other Oomycete plant pathogens (Table 1). These results indicate that the primer/probe set designed specifically amplified *P. medicaginis* DNA.

Results based on the analysis of bulked plant samples indicate that the assay can be used to discriminate between alfalfa populations with different levels of resistance based on the amount of pathogen DNA detected in randomly bulked plant samples (Table 2). The Spearman rank correlations between pathogen DNA content in bulked plant samples and the number of susceptible plants in a bulked sample was positive and highly significant in both experiments (Table 2). This was likely the result of less pathogen growth and multiplication occurring in resistant plants.

The standard test for evaluating seeding resistance in alfalfa to *P. medicaginis* requires that three replications of 50–70 plants be scored for disease reaction (Nygaard et al., 1995). In this report, eight replicated bulks of 15 plants were sufficient for using a real-time PCR assay to separate three different alfalfa check populations in a manner that was consistent with separation for resistance to *P. medicaginis* based on disease rating classes (Table 2). Previously, we had determined in repeated experiments that only four bulks of 15 plants were sufficient for using a real-time PCR assay to clearly discriminate among alfalfa check populations for resistance to *A. euteiches* races 1 and 2 (Vandemark et al., 2002). In addition, six bulks of 15 plants were sufficient for separating 17 different alfalfa populations for resistance to *A. euteiches* race 1 in a manner that was highly consistent with separation based on disease rating classes (Vandemark et al., 2002). The standard test for evaluating seeding resistance in alfalfa to *A. euteiches* requires that four replications of 50–70 plants be scored for disease reaction. The results presented in this report and previously, in the case of *A. euteiches* (Vandemark et al., 2002), suggest that less plants need to be evaluated using real-time PCR assays to realize levels of discrimination among different populations similar to the use of standard tests that rely on visual assessment of disease reaction (Nygaard et al., 1995; Fitzpatrick et al., 1998).

The real-time PCR assay was used to quantify the amount of pathogen DNA in roots of individual plants classified as either resistant or susceptible based on visual examination of disease severity. In both experiments significantly less pathogen DNA was detected in the roots of resistant plants than in susceptible plants (Table 3). These results are similar to those obtained previously based on the use of hyphal staining to attempt to quantify colonization of *P. medicaginis* in alfalfa (Miller and Maxwell, 1984) and chickpea (Dale and Irwin, 1991). However, the real-time fluorescent PCR assay described in this report allows for higher throughput and considerably more precise quantification of the pathogen in infected roots than is possible with hyphal staining. The entire process of DNA extraction, DNA quantification, and running the real-time PCR assay can be completed in 4–5 h. This contrasts with hyphal staining techniques, which require at least 2 days for completion (Miller and Maxwell, 1984).

Significant differences were observed in the amount of pathogen DNA detected in entire root systems of individual plants that were classified as resistant based on visual examination of disease severity. In several

cases, plants were identified in which no pathogen was detectable with the PCR assay. Although it is possible these plants represent escape events in which inoculations failed, this is unlikely given that each plant was inoculated with 250 zoospores. The standard test for evaluating seedling resistance in alfalfa to *P. medicaginis* (Nygaard et al., 1995) calls for inoculating plants with only 50 zoospores, but we chose to increase the amount of inoculum in an attempt to minimize the likelihood of escapes. Accordingly, it is possible that the resistant plants in which no pathogen DNA was detected may be immune to infection by *P. medicaginis*.

The results suggests that the real-time fluorescent PCR assay may be used to select the most resistant plants among a sample of plants that are indistinguishable based on the visual assessment of disease severity. In this study the entire root system of a plant was used for DNA isolation. Sufficient DNA for at least 20 quantitative PCR reactions could be isolated from every single plant, including all plants classified as susceptible. Currently we are conducting seed increases in field isolation cages with over 100 plants that were selected based on results using the real-time PCR assay specific for *A. euteiches* (Vandemark et al., 2002). These plants were assayed by taking a root sample from each plant for DNA extraction while maintaining enough of a root system so that the selected plants could be replanted and maintained for use as breeding material. An alternative approach for maintaining plants that have had their root systems excised for DNA extractions might be to make vegetative cuttings of plants prior to excising root systems.

Several reports suggest that a disease complex of alfalfa seedlings exists that involves both *A. euteiches* and *P. medicaginis* (Havey and Grau, 1985; Holub and Grau, 1990; Munkvold and Carlton, 1995; Wiersma et al., 1995). Many alfalfa breeding programmes simultaneously screen seedlings for resistance to both *P. medicaginis* and *A. euteiches*. It should be possible to use the real-time PCR assay for *P. medicaginis* in combination with the real-time PCR assay for *A. euteiches* (Vandemark et al., 2002) to accurately identify plants with high levels of resistance to both pathogens. It will be necessary to evaluate the utility of selection based on use of real-time PCR assays by comparing the degree of population improvement observed using this strategy with that realized through selection based on visual assessment of disease reaction.

The real-time PCR assays will also be useful for studying factors that influence microbial population dynamics in roots simultaneously or serially infected with both pathogens. The alfalfa populations WAPH-1 and Saranac, are the highly resistant and susceptible standard alfalfa checks respectively for both *P. medicaginis* (Nygaard et al., 1995) and *A. euteiches* race 1 (Fitzpatrick et al., 1998). These two populations could be used for studying interactions between *P. medicaginis* and *A. euteiches* in resistant and susceptible plants with real-time fluorescent PCR assays. The precision and specificity of both real-time PCR assays towards their respective target genomes will provide a novel and accurate approach for studying microbial population dynamics in mixed plant infections.

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